DOXYCYCLINE INHIBITS VASCULAR LEAKAGE AND PREVENTS THE DEVELOPMENT OF PULMONARY EDEMA

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 2008	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Doxycycline Inhibits Vascular Leakage And Prevents The Development Of Pulmonary Edema	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Vascular Biology Program at Childrens Hospital Boston, Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115, U.S.A.**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**4**	

ABSTRACT

The Vascular Leak Syndrome induced by blast injuries, burns, asphyxiation, and other injuries lead to progressive pulmonary edema, as well as tissue and limb swelling that contribute to morbidity and mortality of soldiers on the battlefield. The main goal of our research is the development and validation of novel therapies to prevent and reverse vascular leak syndromes. Our findings indicate that doxycycline, a commonly used antibiotic, inhibits vascular leak in a spectrum of pathologies.

1. INTRODUCTION

The endothelium lining blood vessels serves as a barrier against vascular hyperpermeability, and its maintenance is critical to organ health. Inflammatory mediators evoke tissue edema by disrupting the expression of membrane junctional proteins, which mediate binding between endothelial cell membranes. Endothelial cell-cell junctions form a diffusion barrier between the intravascular and interstitial space. To prevent the morbidity and mortality caused by exaggerated vascular permeability associated with pathologic states (e.g., inflammatory and hypersensitivity disorders, pulmonary edema, traumatic lung injury, cerebral edema resulting from stroke and others), it is important to develop therapeutic approaches to stabilize these inter-endothelial junctions (Fainaru; Adini et al. 2008).

Tetracycyline antibiotics are also potent inhibitors of the matrix metalloproteinase (MMP) proteins and have been used to reduce tissue degradation in arthritis and periodontal disease(Golub; Lee et al. 1998). Doxycycline, a tetracycline derivative, has been shown to inhibit angiogenesis in both humans and animal models; however, its antiangiogenic effect is MMP independent in vitro. We now tested the effect of this FDA-approved oral angiogenesis inhibitor on vascular permeability in mouse models of pulmonary edema and allergic skin reactions.

2. SELECTED METHODS

2.1 Miles vascular permeability assay

C57Bl/6J mice were treated with oral doxycycline (intragastric gavage) at the specified doses or vehicle for 3-5 days before the Miles assay was performed (Claffey; Brown et al. 1996: Miles; Miles 1952: Streit; Velasco et al. 2000). Of note, as it has been previously shown (Prall; Longo et al. 2002) that a doxycycline dose of 100 mg/kg/day achieved a mean plasma concentration similar to plasma levels of human patients taking the recommended dose of 200 mg / day. We therefore used a similar dosing range in all our *in -vivo* experiments. For the Miles assay, Evans blue dye (100 µl of a 1% solution in 0.9% NaCl) was injected intravenously (i.v.) into mice. Evan's blue dye binds to plasma proteins and leaks with them at sites of vessel permeability. After 10 min, 50 µl of human $VEGF_{165}$ (1 ng/µl), PAF (100 µM), histamine (1.2 µg/ml), or PBS was injected intradermally into the preshaved back skin. After 20 min, the animals were sacrificed, and an area of skin that included the entire injection site was removed. Evans blue dye was extracted from the skin by incubation with formamide for 5 days at room temperature, and the absorbance of extracted dye was measured at 620 nm.

2.2 DTH reactions

DTH reactions were induced in the ears of 8-week-old C57Bl/6J male mice (n = 5) as previously described(Dvorak; Lett-Brown et al. 1984). Mice were sensitized by topical application of 2% oxazolone solution in vehicle (acetone:olive oil, 4:1 vol/vol), to the shaved abdomen (50 µl). Mice were treated with oral doxycyline (80 mg/kg/day) for 5 days beginning on day 3, and after 5 days the right ears were challenged by topical application of 10 µl of a 1% oxazolone solution; the left ears were treated with vehicle alone. Ear thickness was then measured daily as a measure of inflammation intensity(Gad; Dunn et al. 1986). Some mice from each experimental group were sacrificed 24 hr after oxazolone challenge. Their ears were fixed in 10% formalin and processed for H&E-stained paraffin sections.

2.3 IL-2-associated pulmonary edema

Mice were pretreated with oral doxycycline (80 mg/kg/day) or vehicle for 5 days. On the sixth day, mice received an intraperitoneal injection with IL-2 (1.2×10^6 units/100 µl) or saline three times a day for 5 days. Doxycycline or vehicle treatment was continued through the course of IL-2 injections. At termination, mice were sacrificed, and lungs were dissected, weighed, fixed, and processed for H&E staining.

2.4 Permeability of endothelial monolayers

Endothelial permeability was analyzed *in vitro* by the diffusion of 2,000-kDa FITC-dextran through the endothelial monolayer (Chen; Pogue et al. 2006). Human microvascular endothelial cells (HMVECs) were grown on Transwell inserts (Costar, Cambridge, MA) up to confluence. The cells pretreated with doxycycline (20 µM) for 16 hours. Medium containing 2.5 mg/ml 2,000-kDa FITC-dextran (Sigma, Sigma-Aldrich Inc, St.Louis , MO) was then loaded in the upper compartment of the Transwell. The amount of FITC-dextran diffused through the endothelial monolayer into the lower compartment was

measured by a microplate reader (Vactor^3, PerkinElmer, Waltham, MA).

3. RESULTS

We first demonstrated (Fainaru; Adini et al. 2008) that doxycycline inhibited VEGF-induced vascular permeability in a dose-dependent manner using the *in vivo* vascular permeability (Miles) assay. This effect was comparable to that achieved by Bevacizumab (Avastin), a powerful inhibitor of VEGF action (Wedam; Low et al. 2006), in the same assay. When compared to tetracycline and other related compounds (i.e minocycline, chloro-tetracycline), doxycycline was the most effective at preventing Evan's blue dye leakage.

In light of doxycycline's ability to prevent vascular permeability, we tested mouse models of clinical conditions where vascular leak serves a major source of morbidity. Immunotherapy with IL-2 represents an important modality in the management of human metatstatic renal cell carcinoma and malignant melanoma, however, this effective treatment is often limited by myriad complications, mainly due to a vascular leak syndrome resulting in pulmonary edema (Berthiaume; Boiteau et al. 1995). To explore the possibility that doxycycline may prevent this potentially lethal complication, mice were pretreated with oral doxycycline (80 mg/kg/day) or vehicle for four days before administering intraperitoneal IL-2 for five days in the continued presence of drug. IL-2 treated mice developed severe pulmonary edema, as demonstrated by a 2.75 fold increase in wet lung weight when compared to vehicle treated controls. Histological sections of the lungs from control mice revealed severe congestion and edema with intra-alveolar fibrin deposition, as well as perivascular and peribronchial mononuclear cell infiltrates. Impressively, doxycycline almost completely inhibited the IL-2 induced increase in lung weight and prevented tissue edema, without producing any evidence of systemic toxicity or weight loss. Our results thus show that IL-2 induced pulmonary edema may be effectively prevented by oral administration of the FDA-approved drug, doxycycline.

The delayed type hypersensitivity reaction (DTH) is also characterized by enhanced vascular permeability and edema formation (Asherson; Ptak 1968), and thus we tested whether doxycycline can inhibit this reaction in a mouse model of contact dermatitis. Mice were sensitized by applying the hapten oxazolone to their abdominal skin, and then were treated either with oral doxycycline (80 mg/kg/day) or vehicle beginning on day 3 after sensitization. Six days after sensitization we challenged the mice by application of oxazolone or vehicle to the right and left ears respectively. Mice treated with doxycycline exhibited significantly reduced ($p < 0.05$) erythema and ear swelling compared with vehicle-treated control mice at 24, 36 and 48 hours. Doxycycline may thus prove to be of value in treating allergic conditions also in humans.

Of the molecular structures comprising the endothelial cell-cell contacts, the adherens junctions, composed of cadherins and catenins, are the primary adhesions between the cells and they are essential for barrier integrity (Lampugnani; Dejana 1997). At the endothelial adherens junction, the key transmembrane protein is VE-cadherin, which clusters together in these regions and mediates cell-cell adhesion through homophilic binding to other VE-cadherins expressed on adjacent endothelial cells (Gumbiner 1996: Lampugnani; Resnati et al. 1992). Paracellular permeability induced by inflammatory mediators, such as VEGF, is accompanied by disruption of the VE-cadherin/catenin complex and loss of cadherin from the cell borders. In fact, disruption of cadherins within the adherens junction mediates the increase in permeability and lung edema that are induced by inflammatory stimuli. Our results show that doxycycline treatment increases the expression of VE-cadherin at the intercellular junctions of human dermal endothelial cells *in vitro*, without significantly altering the expression of β catenin or ZO-1. This effect on VE-cadherin expression may be responsible for the increased barrier function of the endothelium and the decreased protein leak observed *in vivo*.

4. CONCLUSIONS

Taken together, our results indicate that doxycycline may prove useful as a potent oral anti-vascular permeability drug. The mechanism for this effect appears to be upregulation of VE-cadherin at the adherens junctions, thereby enhancing intercellular adhesion and improving the barrier function of the endothelium. Data from these mouse studies may serve as basis for future translation of this therapy into clinical testing. The use of this oral FDA-approved drug to prevent or treat pulmonary vascular leak syndromes as a result of trauma or other injuries sustained during combat, may allow rapid self-treatment by soldiers or other wounded personnel, and might potentially be used as prophylaxis before entering into battle.

5. ACKNOWLEDGEMENTS

This research was supported by the Fulbright and Rothschild Foundations and the European Molecular Biology Organization (EMBO) Fellowship (O.F), Department of Defense Award W81XWH-05-1-0115 (to J.F.).

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